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USE OF RING-DISC ELECTRODES AND VIOLOGENS FOR THE TITRATION OF CYTOCHROME *C* AND OXYGEN AND FOR THE STUDY OF THEIR REDUCTION KINETICS *

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ABSTRACT

The use of ring-disc electrodes enable the electrogeneration of viologen cation radicals $V^{\cdot+}$ when the disc potential is controlled in the range —0.3 to —0.65 and —0.2 to —0.45 V/NHE respectively in MV^{2+} and BV^{2+} solutions. We confirmed the occurrence of very fast reactions between $V^{\cdot+}$ and horse-heart cytochrome *c* or oxygen and of slow or no reaction between $V^{\cdot+}$ and β-nicotinamide adenine dinucleotide with xanthine oxidase, riboflavine and ferricyanide. Ring current versus disc current curves enable the titration of cytochrome *c* and oxygen and the estimation of the reaction rate between cytochrome *c* and $MV^{\cdot+}$ [$(6.2 \pm 2)\ 10^5 M^{-1}\ s^{-1}$].

(1) INTRODUCTION

Electrochemically generated methyl-viologen cation radical ($MV^{\cdot+}$) is known to react rapidly with several oxidizing agents of biological interest. The reaction rate between $MV^{\cdot+}$ and horse-heart ferricytochrome *c* is reported to be in the range $0.5—1 \times 10^6\ M^{-1}\ s^{-1}$ when determined spectroelectrochemically [1—3] and to be in the range $10^8—10^9\ M^{-1}\ s^{-1}$ when determined by pulse radiolysis [4,5]. In a recent spectroelectrochemical work, Mackey and Kuwana [6] report a rate constant greater or equal to $2.0 \times 10^8\ M^{-1}\ s^{-1}$ for this reaction between $MV^{\cdot+}$ and ferricytochrome *c* and a rate constant greater than $4 \times 10^7\ M^{-1}\ s^{-1}$ for the reaction between $MV^{\cdot+}$ and fully oxidized cytochrome *c* oxidase. $MV^{\cdot+}$ reacts also very rapidly with molecular oxygen; according to Leest [7], the stoechiometry is

$$2\ MV^{\cdot+} + 1/2\ O_2 + H_2O \rightarrow 2\ MV^{2+} + 2\ OH^-$$

and its rate constant is larger than $10^9\ M^{-1}\ s^{-1}$ [8].

Since $MV^{\cdot+}$ can be generated electrochemically and detected at an electrode and reacts very rapidly with ferricytochrome *c*, oxidized cytochrome *c* oxydase

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* In honour of Dr. G.C. Barker's 60th birthday.

and oxygen, the use of ring-disc electrodes and viologen cation radicals $V^{\bullet+}$ (methyl- and benzyl-viologens; $MV^{\bullet+}$ and $BV^{\bullet+}$) may be a convenient method for the titration of these molecules. The radical $V^{\bullet+}$ is generated on the disc by reduction of MV^{2+} or BV^{2+} contained in the solution:

(disc) $V^{2+} + e^{-} \rightarrow V^{\bullet+}$

If the solution is free from chemical which can react with $V^{\bullet+}$ during its transit from disc to ring, the observed ring current I_R is proportional to the monitored disc current I_D. If chemicals such as those listed above are present, $V^{\bullet+}$ reacts before reaching the ring. The shape of the I_R versus I_D titration curves, then, may give two kinds of information: the concentration level of the compound reacting with $V^{\bullet+}$ and the rate of the reaction involved in the titration.

When used as analytical tool, these electrodes enable the detection of concentrations as low as 0.1 μM (titration by nascent bromine of phenol- [9] and sulfur-containing compounds [10]) and when used for kinetic studies they allow the determination of first order rate constants up to 10^5 s^{-1} and second order rate constants up to 10^9 M^{-1} s^{-1} [9—11]. Compared to spectro-electrochemical, pulse radiolysis or stopped-flow techniques, this method offers the great advantage of using stationary conditions, i.e. much simpler electrochemical and recording equipment.

We tried to use these ring-disc electrodes to titrate several oxidizing agents belonging to the electron transfer chain of living systems. We first attempted to check if fast reactions occur between $V^{\bullet+}$ and these compounds. For those which actually react rapidly enough, i.e. oxygen and cytochrome *c*, we recorded I_R versus I_D titration curves and tried to determine the corresponding rate constant by their mathematical analysis.

(2) EXPERIMENTAL

(2.1) Reagents

Water was distilled in a glass vessel. All compounds were used without further purification: methyl- and benzyl-viologens (B.D.H.), horse heart ferricytochrome *c* (Calbiochem, grade A and Boehringer), beef liver catalase (E.C. 1.11.1.6) (Boehringer), riboflavine (Merck), βNAD^+ (Sigma grade III, preweighted vials), milk xanthine oxidase (E.C. 1.2.3.2) (Boehringer), KH_2PO_4 (Prolabo R.P.) and KOH (Prolabo, normadose), $K_3Fe(CN)_6$ (Merck p.a.).

(2.2) Materials

We used the Tacussel bipotentiostat (BIPAD) and potential monitors (Servovit 9 B and GSTP 2) to obtain linear sweeps of the disc potential or of the disc current (the ring being set at a constant potential) or linear sweeps of the ring potential (the disc potential or the disc current being constant). The disc, or the ring potential is measured with respect to an Ag/AgCl, saturated KCl

TABLE 1

Geometrical characteristics of ring-disc electrodes — calculated and measured collection efficiency N_0

	Glassy carbon disc, glassy carbon ring, electrode No. 1	Platinum disc, platinum ring, electrode No. 2
r_1 (radius of disc electrode)/mm	2.82 ± 0.05	1.98 ± 0.05
r_2 (inner radius of the ring electrode)/mm	3.12 ± 0.05	2.12 ± 0.05
r_3 (outer radius of the ring electrode)/mm	3.94 ± 0.05	2.37 ± 0.05
Calculated N_0 [a]	0.369	0.256
Observed N_0	0.370 ± 0.014 [b]	0.269 ± 0.020 [c]
$\alpha = [r_2/r_1]^3 - 1$	0.354	0.227
$\beta = [r_3/r_1]^3 - [r_2/r_1]^3$	1.370	0.487
F(α)	0.541	0.479
$N_0/\beta^{2/3}[1 - F(\alpha)]$	0.655	0.785

[a] Theoretical equation from ref. 11.
[b] Average of 5 experiments with $MV^{2+}/MV^{\bullet+}$ and $BV^{2+}/BV^{\bullet+}$ system.
[c] Average of 8 experiments at ω = 28.6 Hz, $[Br^-]$ = 0.5 *M*, E_A = +0.45 V/NHE with Br_2/Br^- system.

electrode (Tacussel AgCl 10) with an electronic millivoltmeter (Tacussel S6N). The simultaneous recording of two variables among: ring and disc current, ring and disc potential was obtained on an Goertz XY recorder (Servogor). Glassy carbon ring-glassy carbon disc (No. 1) and platinum ring-platinum disc (No. 2) electrodes were constructed by Coquelet (Electricité de France, Chatou). Table 1 gives the characteristics of these electrodes. Diameters have been measured from photographic enlargements where a length reference also appeared (slide calipers opened at 1 ± 0.05 cm).

The rotation speed of the direct-current motor which drove the electrode was monitored (Tacussel Asservitex) at a value usually equal to 23.8 ± 0.1 Hz. The rotation speed was checked with a Jacquet revolution counter.

The 25 ml cell was thermostated at 20.0 ± 0.2°C.

(2.3) Procedure

Stock solutions of viologens were prepared, just before the experiments, by dissolving in 25 ml of 0.2 *M* pH 6.8 phosphate buffer 6.4 or 64 mg of MV^{2+} (1 and 10 m*M*) or 10.2 mg BV^{2+} (1 m*M*). The oxidizing agents (cytochrome *c*, riboflavine, NAD^+ and ferricyanide) in solid form were then added to the purged solution.

The ring-disc electrode surfaces were polished using plastic sheets incoporated with 3 and 0.3 μm diamond (Tacussel BSC 3 and BAO 3) before every sequence of recordings. Repeated sweeps of disc current in a pure buffered

V^{2+} solution allow us to obtain a reproducible I_R versus I_D blank straight line which is used as a reference for the titration curves. Potential scans were performed at a rate of 250 mV min^{-1} and current scan-rates were chosen so that curves obtained with increasing or decreasing I_D agreed with one another (usually 100 s in each direction). When the disc was monitored for current, the ring potential was adjusted at a suitable value to obtain a low ring current background noise when $I_D = 0$. This ring potential was always high enough to allow the complete oxidation of $V^{\bullet+}$.

Unless special specification, solutions where carefully deoxygenated for at least 15 min before examination and kept under nitrogen atmosphere. Nitrogen used for this purpose was previously freed from traces of oxygen by bubbling through a basic pyrogallol solution and through distilled water. Special plastic tubes (Isoversinic) of low porosity towards oxygen were used to bring nitrogen to the electrochemical cell.

In spite of these conditions, I_R versus I_D blank lines are slightly curved near the origin (Fig. 7, curve 1) and present a difference according to the scan direction of I_D monitoring. This behaviour is probably not related to the presence of traces of oxygen since it has also been observed during titration of phenol- and sulfur-containing compounds by nascent bromine [9,10]. Nevertheless, this artifact becomes negligible on titration curves of rather concentrated solutions (Fig. 5).

For oxygen titration by electrogenerated $V^{\bullet+}$, we mixed two streams of oxygen and nitrogen which flows were adjusted using two flow-meters (Brooks R2 25 B): with this equipment, the partial pressure of oxygen was regulated between 0.1 and 1 atmosphere.

Most current-potential curves were obtained with the glassy carbon ring-disc electrode (No. 1) which gives a very low background current in the —0.7 to 0 V/NHE potential range. I_R versus I_D titration curves were achieved either with this electrode or with the platinum ring-disc electrode (No. 2) and no significant difference was observed.

(3) RESULTS AND DISCUSSION

(3.1) Electrochemical behaviour of viologens on ring-disc electrodes

The first pH-independent reduction step of viologens V^{2+} into the violet cation radical $V^{\bullet+}$ is clearly reversible (Table 2):

$E^0 = -0.45 \pm 0.02$ V/NHE for $MV^{2+}/MV^{\bullet+}$

$E^0 = -0.33 \pm 0.03$ V/NHE for $BV^{2+}/BV^{\bullet+}$

This electrochemical reversibility is illustrated in Fig. 1 with the $MV^{2+}/MV^{\bullet+}$ system. In a MV^{2+} solution, the disc current is set at a negative value . MV^{2+} is reduced into $MV^{\bullet+}$ and this radical may be detected on the ring by its electrochemical reoxidation into MV^{2+}. Thus, on the ring current-potential curves

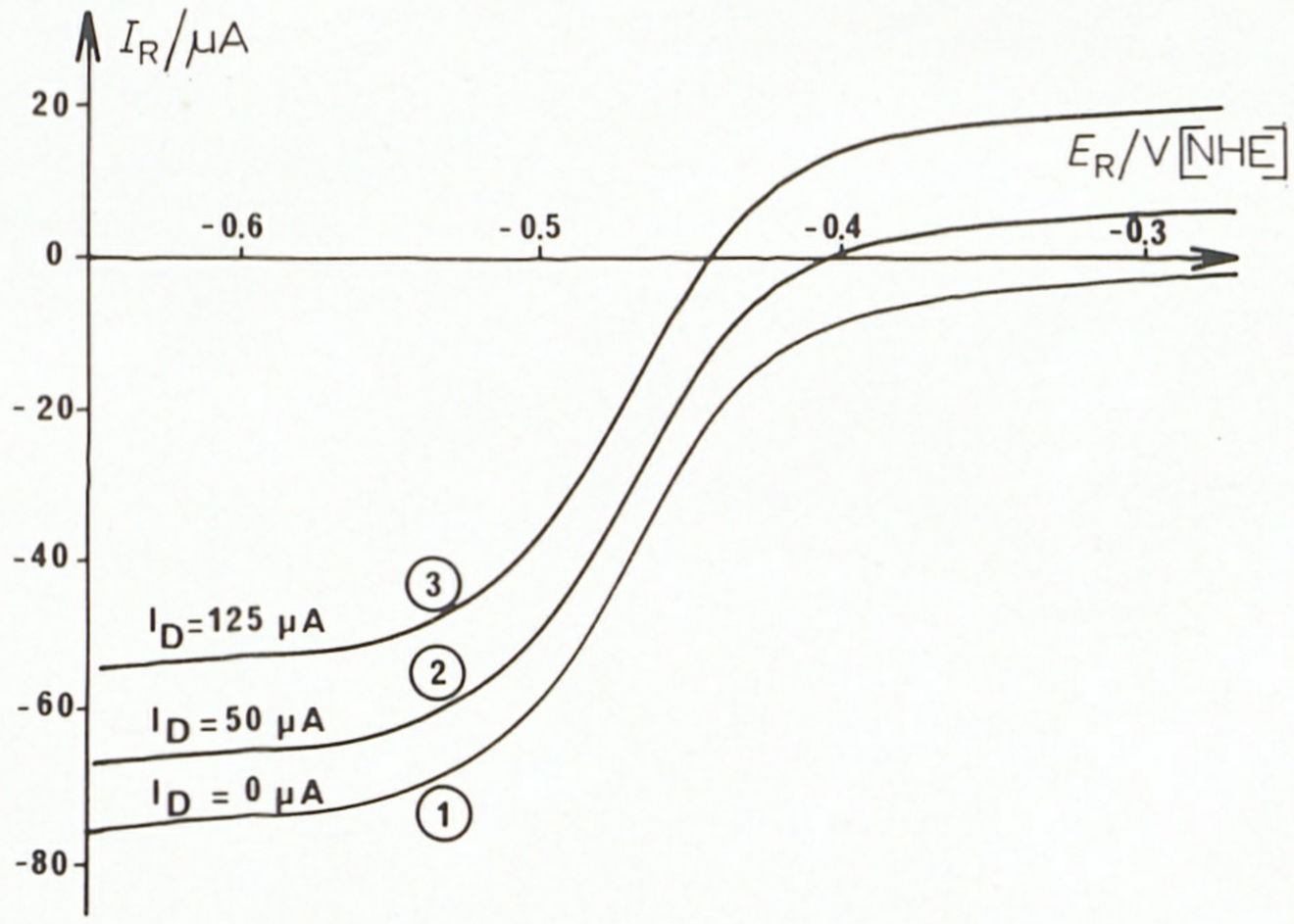

Fig. 1. Reversibility of $MV^{2+}/MV^{\bullet+}$: influence of the monitored disc current on the ring voltammetric curves in a 1.1 m*M* MV^{2+} solution. Phosphate buffer (pH 6.8), electrode No. 1. Monitored disc current value: (1) 0 μA, (2) 50 μA, (3) 125 μA. Limiting value of the disc current for the first MV^{2+} reduction wave: 115 ± 5 μA.

achieved in the —0.45 V region, we observe an anodic current proportional to the cathodic disc current monitored; the decrease of the cathodic ring current in the —0.6 V region is related to the depletion of MV^{2+} near the electrode due to its reduction on the disc. Identical curves have been obtained with BV^{2+} solutions in the —0.36 V region. So, if the ring potential is set at a constant value, greater than the above mentioned $V^{2+}/V^{\bullet+}$ standard potentials, a slow decrease of the disc potential gives simultaneously a disc current-disc potential cathodic wave (V^{2+} electrochemical reduction) and a ring current-disc potential anodic wave ($V^{\bullet+}$ electrochemical oxidation) (Fig. 2 and curves 1D and 1R of Fig. 4). Finally, with the same ring potential, monitoring the disc current gives I_R versus I_D curves (Fig. 5, curve 1) which are straight lines, the slope of which is equal to the calculated collection efficiency N_0 (Table 1).

The reversibility of the first reduction step of BV^{2+} is also demonstrated by the electrochemical properties of $BV^{\bullet+}$ prepared by chemical reduction of BV^{2+} by dithionite. To a 1 m*M* BV^{2+} solution buffered at pH 6.8, we added aliquots of 4 m*M* dithionite solution; voltammograms obtained at rotating platinum or glassy carbon disc electrodes show that the first reduction wave of BV^{2+} is gradually replaced by the anodic wave of $BV^{\bullet+}$ while the solution turns violet; the half-wave potentials of both waves are identical and the sum of their heights is independent of the amount of dithionite added.

In contrast to the $V^{2+}/V^{\bullet+}$ system, the second reduction step of V^{2+}, i.e. $V^{\bullet+} \rightarrow V^0$, is not electrochemically reversible. Figure 3 shows the modification of the curves presented in Fig. 2 when the disc potential is monitored to more negative potentials. On the cathodic potential scan (continuous lines), a second wave appears on the disc current-potential curve (1D) indicating a

TABLE 2

Electrochemical properties of 1 m*M* viologen solutions in phosphate buffer at pH 6.8

Electrode	$MV^{2+}/MV^{\bullet+}$ red. of MV^{2+} ox. of $MV^{\bullet+}$ *E*/V(NHE)	$MV^{\bullet+}/MV^{0}$	
		red. of $MV^{\bullet+}$ *E*/V(NHE)	ox. of MV^{0} *E*/V(NHE)
Rotating glassy carbon disc	−0.46 ± 0.01	−0.80 ± 0.01	−0.66 [a]
Rotating platinum disc	−0.45 ± 0.01		
	−0.446 [c]		
Stationary platinum disc (0.1 V s^{-1})			
Rotating gold disc			
Dropping mercury electrode			
	−0.433 ± 0.002 [e]	−0.793 [e]	
Hanging mercury drop (0.1 V s^{-1})			

[a] Potential of the peak obtained on return scan (Fig. 3).
[b] In glycine buffer (pH 9.9).
[c] From Michaelis [13].
[d] From Leduc [12].
[e] From Elofson [14], pH 5—13.

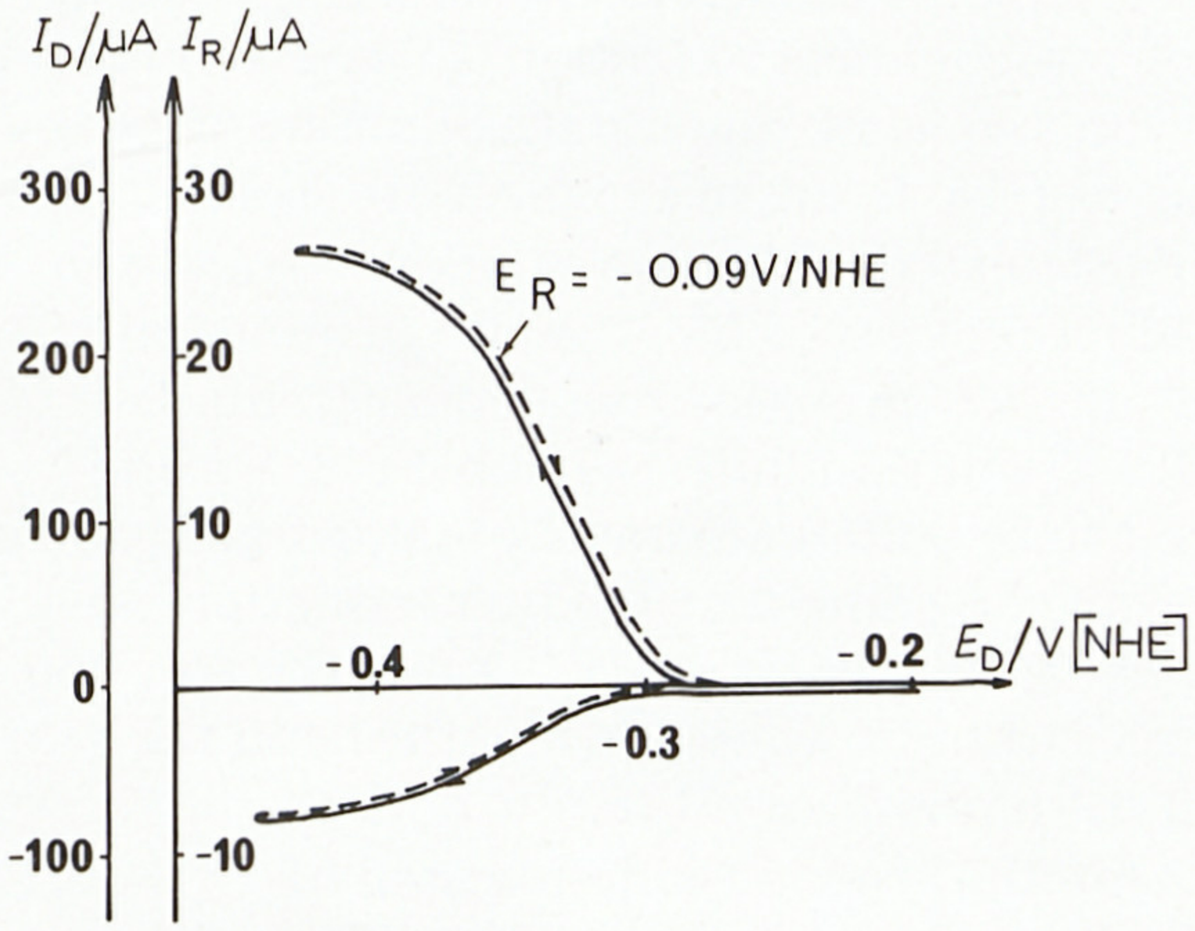

Fig. 2. Formation and detection of $BV^{\bullet+}$ in a 1 m*M* BV^{2+} solution. Phosphate buffer (pH 6.8), electrode No. 1, monitored ring potential −0.09 V/NHE. (———) normal scan: curves obtained with decreasing disc potential; (- - - - -) reverse scan: curves obtained with increasing disc potential.

$BV^{2+}/BV^{\bullet+}$ red. of BV^{2+} ox. of $BV^{\bullet+}$ E/V(NHE)	$BV^{\bullet+}/BV^{0}$	
	red. of $BV^{\bullet+}$ E/V(NHE)	ox. of BV^{0} E/V(NHE)
—0.31 ± 0.02	—0.46 ± 0.02	—0.32 [a]
—0.31 ± 0.02 [b]	—0.46 ± 0.02 [b]	
—0.31 ± 0.02 [b]	—0.47 ± 0.02 [b]	
—0.37 ± 0.02 [b,d]	—0.53 ± 0.02 [b,d]	
—0.359 [c]		
E_p^c = —0.38 ± 0.02 [b,d]		
E_p^a = —0.30 ± 0.02 [b,d]		
—0.35 ± 0.01 [b,d]		
—0.33 ± 0.02	—0.50 ± 0.02	
—0.35 ± 0.02 [b]	—0.50 ± 0.02 [b]	
—0.365 ± 0.01 [b,d]	—0.523 ± 0.01 [b,d]	
—0.347 ± 0.005 [e]	—0.537 [e]	
E_p^c = —0.35 ± 0.02 [b,d]	E_p^c = —0.52 [b,d]	E_p^a = —0.37 [b,d]
E_p^a = —0.30 ± 0.02 [b,d]		

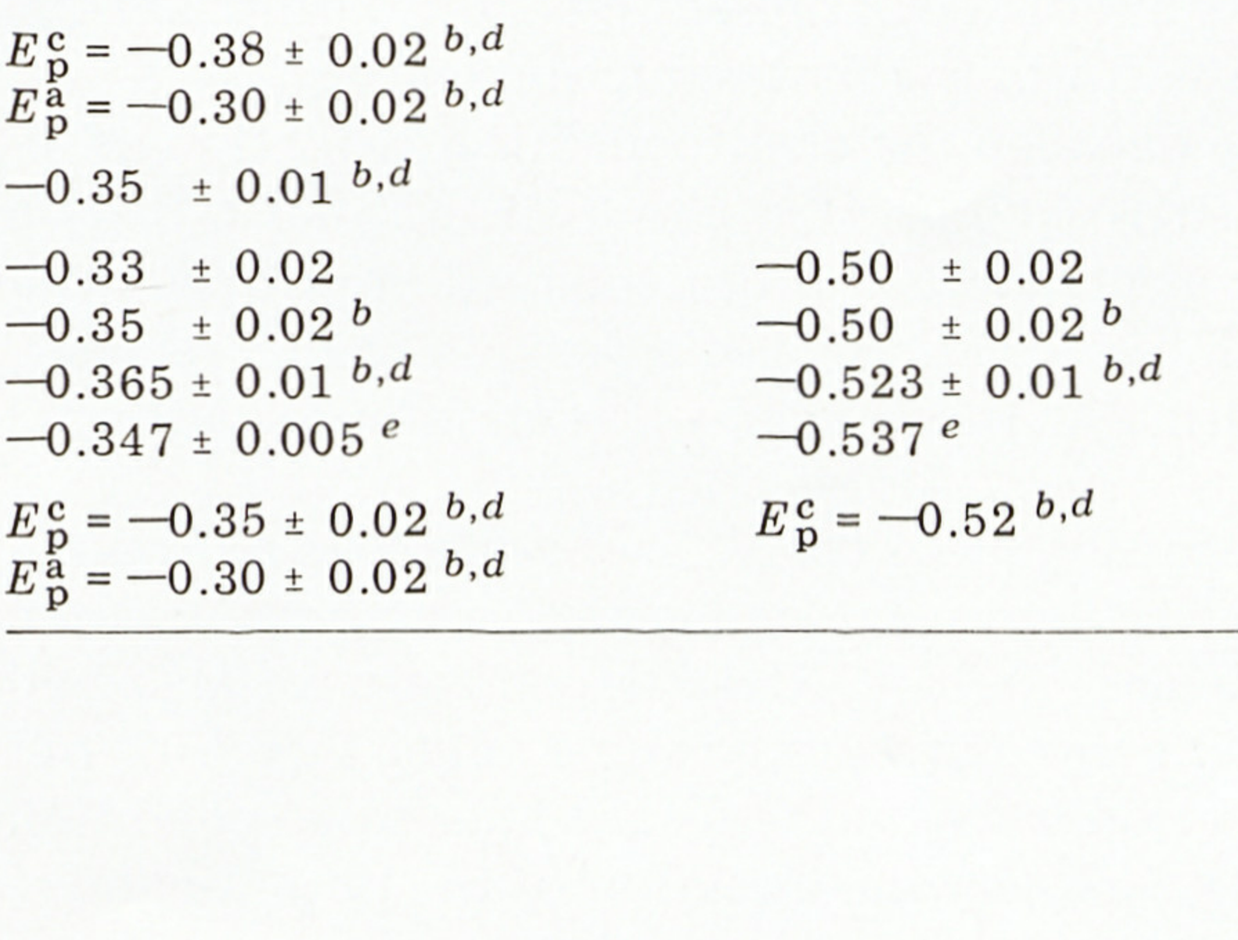

Fig. 3. Formation and detection of $BV^{\bullet+}$ and BV^{0} in a 1 m*M* BV^{2+} solution. Phosphate buffer (pH 6.8), electrode No. 1, monitored ring potential —0.09 V/NHE. (———) normal scan: curves obtained with decreasing disc potential; (- - - - - -) reverse scan: curves obtained with increasing disc potential.

second pH-independent electron transfer:

$$BV^{\bullet+} + e^- \rightarrow BV^0$$

Simultaneously, the ring current decreases (1R) indicating the disappearance of $BV^{\bullet+}$ near the ring electrode and the quasi absence of electroactive compound reaching the ring. After an inversion of the potential scan at —0.65 V, the curves obtained on the return scan (dotted lines) are very different from the previous ones. Instead of the two cathodic waves, disc current-potential curve (1′D) presents a single anodic peak (E_p = —0.32 V/NHE) sometimes accompanied by a shoulder at —0.30 V/NHE. This peak is also observed on the ring current-disc potential curve (1′R) (E_p = —0.32 V/NHE). The same phenomena were observed with methyl viologen: the second reduction wave occurs at —0.80 V/NHE and the anodic peak present on the return scan (E_p = —0.66 V/NHE) is well separated from the first reduction wave ($E_{1/2}$ = —0.46 V/NHE). This difference between the electrochemical reduction of $V^{\bullet+}$ and oxidation of V^0 (Table 2) suggests that V^0 is either strongly adsorbed on the electrode or poorly soluble at pH 6.8 and thus deposited on the electrode as soon as it is electrogenerated. The occurrence of a peak on the I_R versus E_D curves obtained on the return scan shows that $V^{\bullet+}$ is an intermediate in the oxidation of V^0 into V^{2+}.

Taking into account this irreversibility of the $V^{\bullet+}/V^0$ system on platinum and glassy carbon electrodes, we generally avoided monitoring the disc potential at a value lower than —0.65 and —0.45 V/NHE, respectively for MV^{2+} and BV^{2+} solutions, or monitoring the disc current at a value larger than 90% of the height of the V^{2+} first reduction wave on the disc. Under these conditions, all recordings were reproducible whatever the direction of the potential or current scan was.

(3.2) Titrations

Oxygen. As shown in Fig. 4 there is a fast reaction between $MV^{\bullet+}$ and dissolved oxygen: indeed $MV^{\bullet+}$ generated on the disc may in some conditions be undetectable on the ring because of its reaction with oxygen present in the solution. In a deoxygenated MV^{2+} solution, we observe the usual formation of $\dot{M}V^{\bullet+}$ on the disc (curve 1D) and its detection on the ring (1R). In an air-saturated solution where MV^{2+} is absent, oxygen is reduced on the disc at about —0.2 V/NHE (curve 2D) but no H_2O_2 is detected on the ring if its potential is set at +0.2 V/NHE (2R). Now, if both components are present, i.e. 0.29 m*M* O_2 and 1 m*M* MV^{2+}, the voltammetric curve on the disc (curve 3D) is exactly the sum of the two former, whereas the ring current remains zero (3R). Thus, if the disc potential is monitored at a value lower than —0.4 V, $MV^{\bullet+}$ generated on the disc cannot reach the ring and give an anodic current for it is entirely reoxidized by the dissolved oxygen. In terms of disc current, neither low $|I_D|$ which corresponds to the reduction of oxygen, nor high $|I_D|$ which corresponds to the generation of $MV^{\bullet+}$ consummed by oxy-

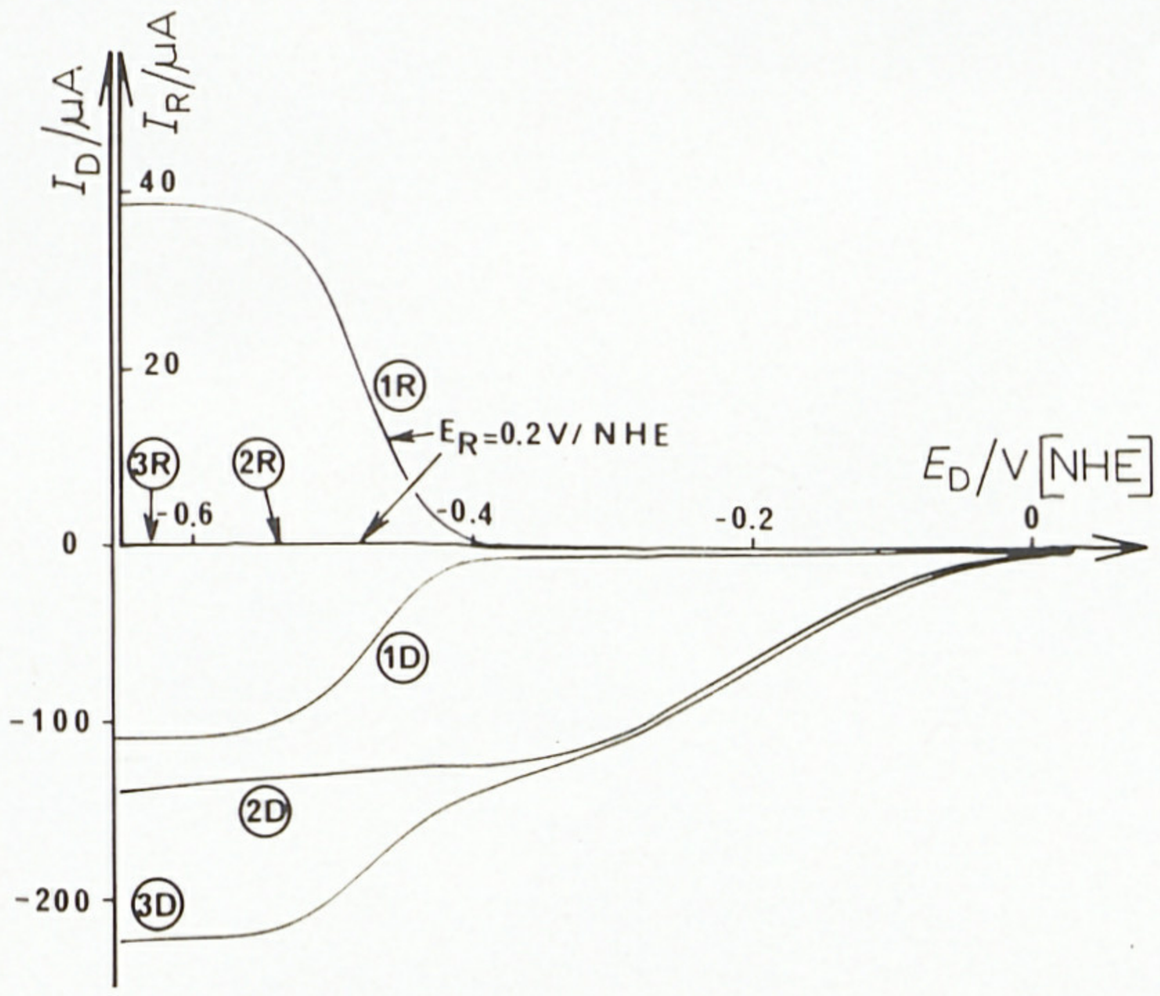

Fig. 4. Reactivity of $MV^{\bullet+}$ towards dissolved oxygen. Phosphate buffer (pH 6.8), electrode No. 1, monitored ring-disc potential +0.2 V/NHE. Concentration of methyl viologen: (1) and (3) 1 m*M*, (2) 0 m*M*. Concentration of oxygen: (1) 0 m*M*, (2) and (3) 0.29 m*M*, i.e., air saturated at 20°C.

gen as soon as it is formed, give any ring current. We have shown by a similar set of curves that $BV^{\bullet+}$ reacts very rapidly with dissolved oxygen.

Before presenting the analytical use of such fast reactions between oxygen and $V^{\bullet+}$, we wish to point out that the analysis of I_R versus I_D curves like those presented in Fig. 5 does not allow us to know whether the chemical reaction involved is fast or not. If no reaction was occurring between oxygen and $V^{\bullet+}$, we would detect a zero I_R as long as monitored $|I_D|$ is smaller than $|I_{lim}|$, the height of the reduction wave of oxygen on the disc: indeed, H_2O_2 generated by electrochemical reduction of oxygen is not detected on the ring when E_R = +0.2 V/NHE. For higher monitored $|I_D|$, $V^{\bullet+}$ detected on the ring would be proportional to the amount of $V^{\bullet+}$ generated on the disc and the I_R versus I_D curves would have the same shape as those actually obtained (Fig. 5). Thus, it is necessary to check the occurrence of a fast reaction between oxygen and $V^{\bullet+}$ on I_D versus E_D and I_R versus E_D curves (Fig. 4); such a test being achieved, it is noteworthy that I_R versus I_D curves are indifferent to the possibility of electrochemical reduction of oxygen on the disc: as soon as a sufficient flux of $V^{\bullet+}$ is generated on the disc, oxygen concentration is zero on the disc electrode and everything proceeds as if oxygen was not electroactive [11].

In another group of experiments we have increased the V^{2+} concentration to 10 m*M* and regulated the partial pressure of oxygen above the solution. The I_R versus I_D titration curves (Fig. 5) present the usual characteristics expected for fast second order reactions i.e. a zero I_R at low I_D values and an asymptotical part at high I_D values. But the distance between the I_R versus I_D line

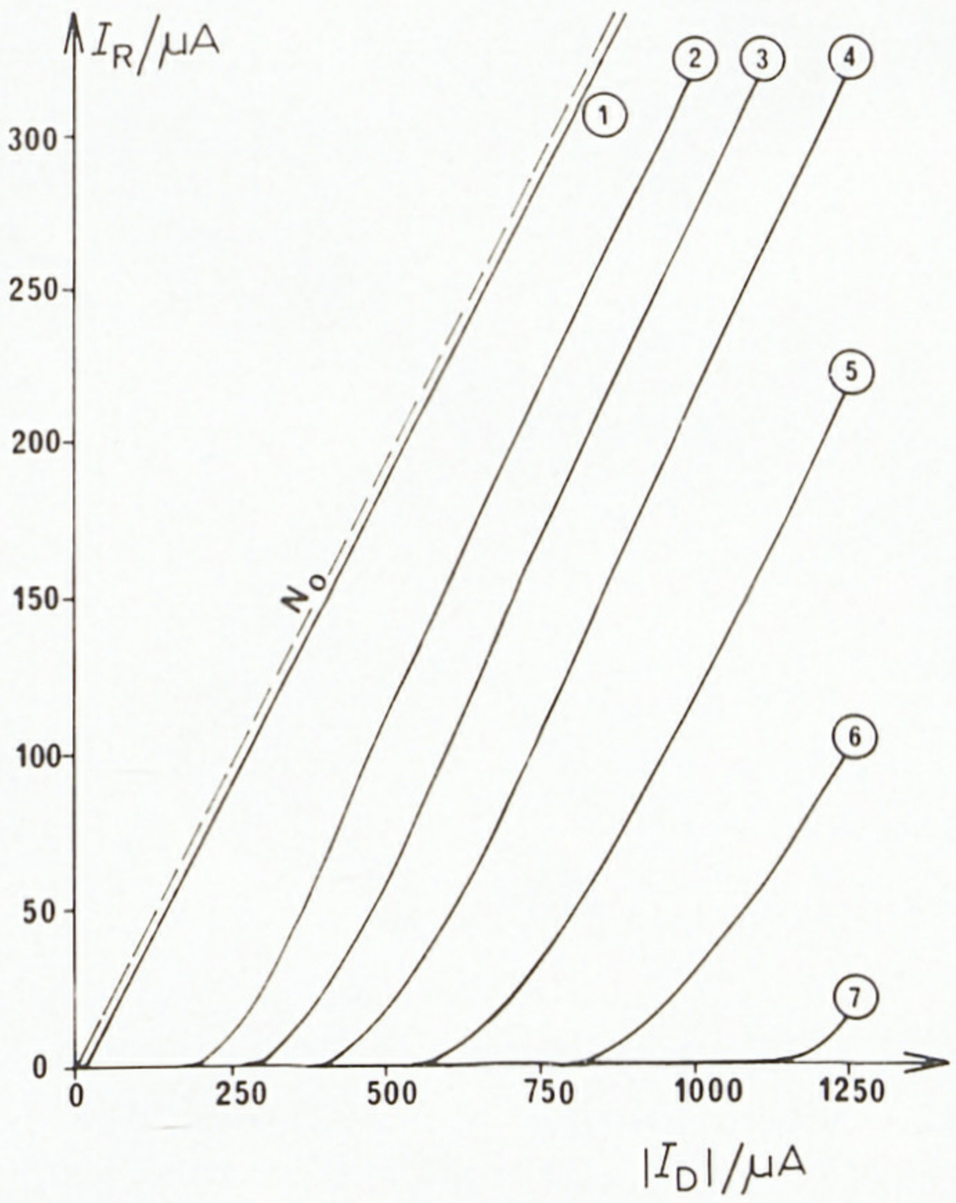

Fig. 5. Oxygen titration curves by electrogenerated $MV^{\bullet+}$: ring current versus disc current curves at different oxygen concentrations. Phosphate buffer (pH 6.8), electrode No. 1, monitored ring potential +0.2 V/NHE. MV^{2+} 10 mM, concentration of oxygen (1) 0, (2) 0.20, (3) 0.35, (4) 0.46, (5) 0.72, (6) 1.0, (7) 1.45 mM; (- - - - - -) line of slope N_0 (Table 1).

obtained with oxygen and the blank straight line of slope N_0, i.e. $I_{D,O} = |I_D| - I_R/N_0$ is not constant as is usually observed for fast chemical reactions [11]. This behaviour is possibly related to the complexity of the reaction between oxygen and $V^{\bullet+}$ involving 4 moles of $V^{\bullet+}$ and probably different reaction rates for the successive steps. Nevertheless, the value of the disc- current at which the ring current becomes different from zero is proportional to the concentration of dissolved oxygen and may be used for its titration. Since the chemical reactions involved are very fast, the lower oxygen concentration which can be measured by this method is probably about 0.1 μM: this assumption was not actually checked for we could not, with our gas equipment, regulate such a low partial pressure of oxygen (0.6×10^{-4} atm). In order to compare this method of titration of oxygen with the usual voltammetric determinations on solid electrodes, we may refer to Figs. 4 and 5: for an air-saturated 0.29 mM oxygen solution the height of the cathodic wave (Fig. 4, curve 2D) equals 120 μA, whereas $I_{D,O}$ on I_R versus I_D curves equals about 250 μA (Fig. 5, interpolation between curves 2 and 3). Thus, the signal to be measured is increased 2 times by using ring-disc electrode titrations of oxygen by $V^{\bullet+}$.

Cytochrome c. As with oxygen, we have confirmed the occurrence of a fast reaction between $V^{\bullet+}$ and horse-heart ferricytochrome *c*, showing that, in the

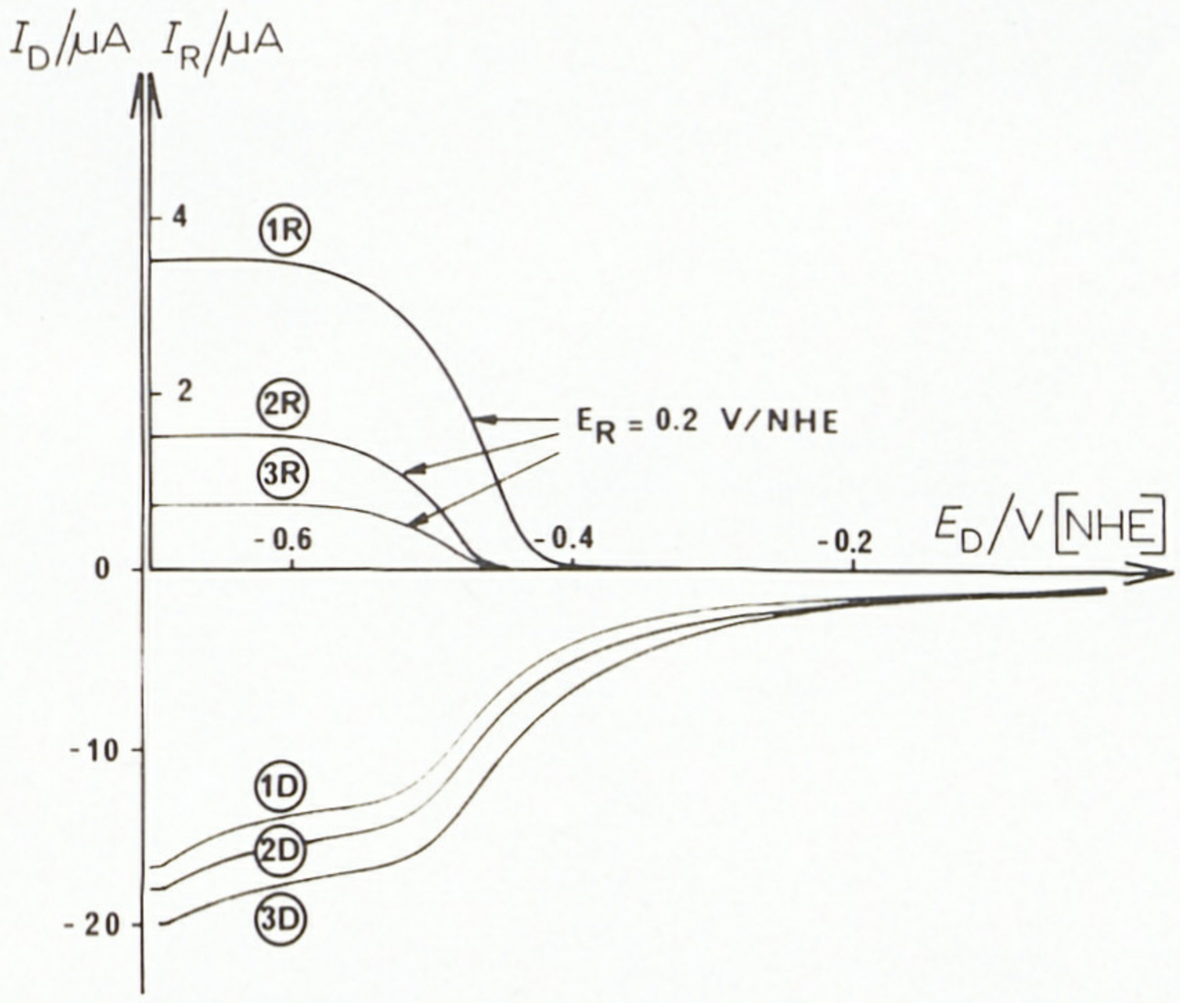

Fig. 6. Reactivity of $MV^{\bullet+}$ towards horse heart ferricytochrome *c*. Phosphate buffer (pH 6.8), electrode No. 1, monitored ring potential +0.2 V/NHE, MV^{2+} 0.1 m*M*, cytochrome *c* (1) 0, (2) 22 and (3) 45 μM.

presence of this compound, $MV^{\bullet+}$ or $BV^{\bullet+}$ can be chemically reoxydized during their transit from disc to ring. Figure 6, obtained with different cytochrome *c* concentrations may be compared to Fig. 4 related to the reaction between oxygen and $MV^{\bullet+}$. As curves 2R and 3R of Fig. 6 differ from the E_D axis, it is clear that with 22 and 45 μM cytochrome *c* solutions the fluxes of $MV^{\bullet+}$ generated on the disc are too large for $MV^{\bullet+}$ to be completely oxidized into MV^{2+} before reaching the ring; it is nevertheless possible to obtain a zero ring current and I_R versus E_D curves identical to curve 3R of Fig. 4: cytochrome *c* concentration have to reach a value about 0.2 m*M*, MV^{2+} still being 0.1 m*M*. When compared to curve 1D, curves 2D and 3D of Fig. 6 show that 22 and 45 μM cytochrome *c* solutions may not be titrated by their electrochemical reduction on a rotating glassy carbon disc electrode: even if this electrochemical reduction occurs, which is not clear from these curves and was not found by Kuwana [6], the titration with ring-disc electrodes is much more sensitive and accurate. Such titration curves are shown in Fig. 7: I_R versus I_D curves present all the characteristics of fast reactions, i.e. zero I_R for $|I_D|$ and an asymptote of slope N_0 for high $|I_D|$ values. As the distance between this asymptote and the blank line, i.e. $I_{D,O} = |I_D| - I_R/N_0$ is proportional to the concentration of cytochrome *c*, the value of $I_{D,O}$ may be used for the determination of an unknown concentration. Such a titration is realized with 3 I_R versus I_D curves:

— the blank line (Fig. 7, curve 1) in absence of cytochrome *c*
— the calibration line (Fig. 7, curves 2, 3 or 4) in the presence of a known concentration of cytochrome *c*;
— the measurement itself with the unknown solution.

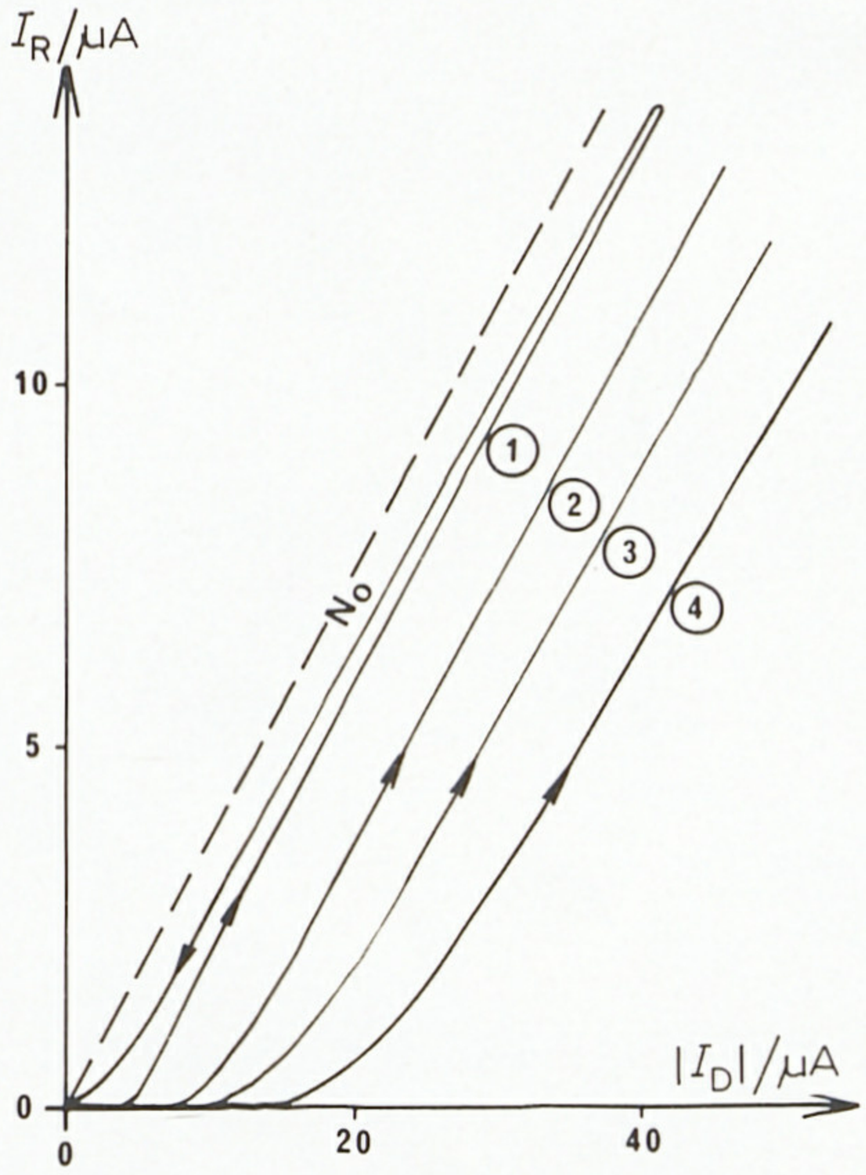

Fig. 7. Horse heart cytochrome *c* titration curves by electrogenerated $BV^{\bullet+}$ ring current versus disc current curves at different cytochrome *c* concentrations. Phosphate buffer (pH 6.8), electrode No. 1, monitored ring potential +0.1 V/NHE, BV^{2+} 1.24 m*M*. Cytochrome *c* (1) 0, (2) 40, (3) 80, (4) 120 μM; (- - - - - -) line of slope N_0 (Table 1).

Apart from the small curvature of the blank line already mentioned it is clear that the sensitivity of the titration is limited by the presence of traces of oxygen and may differ from one equipment to another according to the quality of the deoxygenation and the isolation of the electrochemical cell towards atmosphere. The accuracy and reproducibility of such titrations was found to be about 7% in the 20—300 μM range.

Various oxidizing agents. In order to compare cytochrome *c* and oxygen with other components of the electron transfer chain of living systems, we have studied the reactivity of electrogenerated $V^{\bullet+}$ with β-nicotinamide adenine dinucleotide (NAD^+) and riboflavine.

NAD^+ is known to equilibrate with $BV^{\bullet+}$ in the presence of milk xanthine oxidase [12]: the $BV^{2+}/BV^{\bullet+}$ system is used as mediator in the electrochemical determination of the standard potential of NAD^+/NADH (—0.32 V/NHE at pH 7) [15]. The I_R versus E_D curves obtained with 0.1 m*M* MV^{2+} solution buffered at pH 6.8 and containing up to 0.8 m*M* NAD^+ and 0.4 μM xanthine oxidase, show that, at a time scale of a few milliseconds, no reaction modifies the amount of $MV^{\bullet+}$ reaching the ring. Since the above mentioned chemical equilibrium is indeed reached within 20—30 min, the fact that no reaction is observed is not surprising.

Riboflavine which is a stronger oxidizing agent than NAD^+ (E'_0 = —0.21

V/NHE at pH 7) seems not to give any fast reaction with $MV^{\bullet+}$ as checked on I_R versus E_D curves obtained in a 0.1 m*M* MV^{2+} and 0.1 m*M* riboflavine solution.

In order to illustrate the role of the protoporphyrin and protein moieties of ferricytochrome *c* in the reactivity of its Fe(III) atom with $V^{\bullet+}$, we have studied the properties of ferricyanide. The I_R versus E_D curves obtained with 1 m*M* MV^{2+} solution buffered at pH 6.8 show that addition of a 2.4 excess of ferricyanide decreases the amount of $MV^{\bullet+}$ detected on the ring to less than 50%. Compared to the results presented in Fig. 6, this means that ferricyanide reacts with $MV^{\bullet+}$ but much more slowly than horse-heart ferricytochrome *c*.

(4) KINETIC STUDIES

Reactions of $V^{\bullet+}$ with oxygen and cytochrome *c* being very fast, $V^{\bullet+}$ and these two oxidizing agents cannot exist simultaneously outside of a narrow volume at the border of two regions near the electrode: one where $V^{\bullet+}$ alone is present and one where oxygen or cytochrome *c* alone is present. If these reactions are second-order, determination of the rate constant k_2 from eqn. (a)

$$-d[V^{\bullet+}]/dt = k_2[V^{\bullet+}][C] \qquad (a)$$

(where [C] is the concentration of the compound reacting with $V^{\bullet+}$) is related to the value of the ring current $I_{R,K}$ at which the reaction zone first reaches the ring [11]. The corresponding disc current $I_{D,K}$ is calculated from $I_{D,O}$ by means of eqn. (b) assuming that $V^{\bullet+}$ and C present equal diffusion coefficients

$$I_{D,K} = \{N_0/[\beta^{2/3}(1 - F(\alpha))]\}I_{D,O} \qquad (b)$$

where α and β are geometrical parameters of the electrode (Table 1), F a numerical function of the parameters, $I_{D,O}$ the value of the disc current at which the asymptote crossed the I_D axis and N_0 the collection efficiency. k_2 is then given by:

$$k_2 = 0.339[r_2/r_1]^2(D/\nu)^{1/3}[1 - F(\alpha)](2\pi\omega/[C])(I_{D,K}/I_{R,K}) \qquad (c)$$

where *D* is the diffusion coefficient of $V^{\bullet+}$ (1.3×10^{-5} cm^2 s^{-1}) for MV^{2+} [6], ν the viscosity of the solution ($\nu \simeq 10^{-2}$ cm^2 s^{-1}), ω the rotation speed of the electrode (23.8 Hz).

As I_R versus I_D curves obtained with 10 m*M* MV^{2+} and 0.2 to 1.45 m*M* oxygen solutions, buffered at pH 6.8 (Fig. 5) present abnormal features, i.e. $|I_D| - I_R/N_0$ is not constant for high $|I_D|$ values, it is impossible to apply the above mentioned theory.

On the contrary, the I_R versus I_D titration curves obtained with 10 m*M* MV^{2+} and 20 to 310 μM ferricytochrome *c* solution (Fig. 6) exhibit a well defined asymptote of slope N_0. We estimated the rate of this reaction, assumed to be second-order, by measuring $I_{D,K}$ and $I_{R,K}$ on several I_R versus I_D curves corresponding to cytochrome *c* concentration in the range 70 to

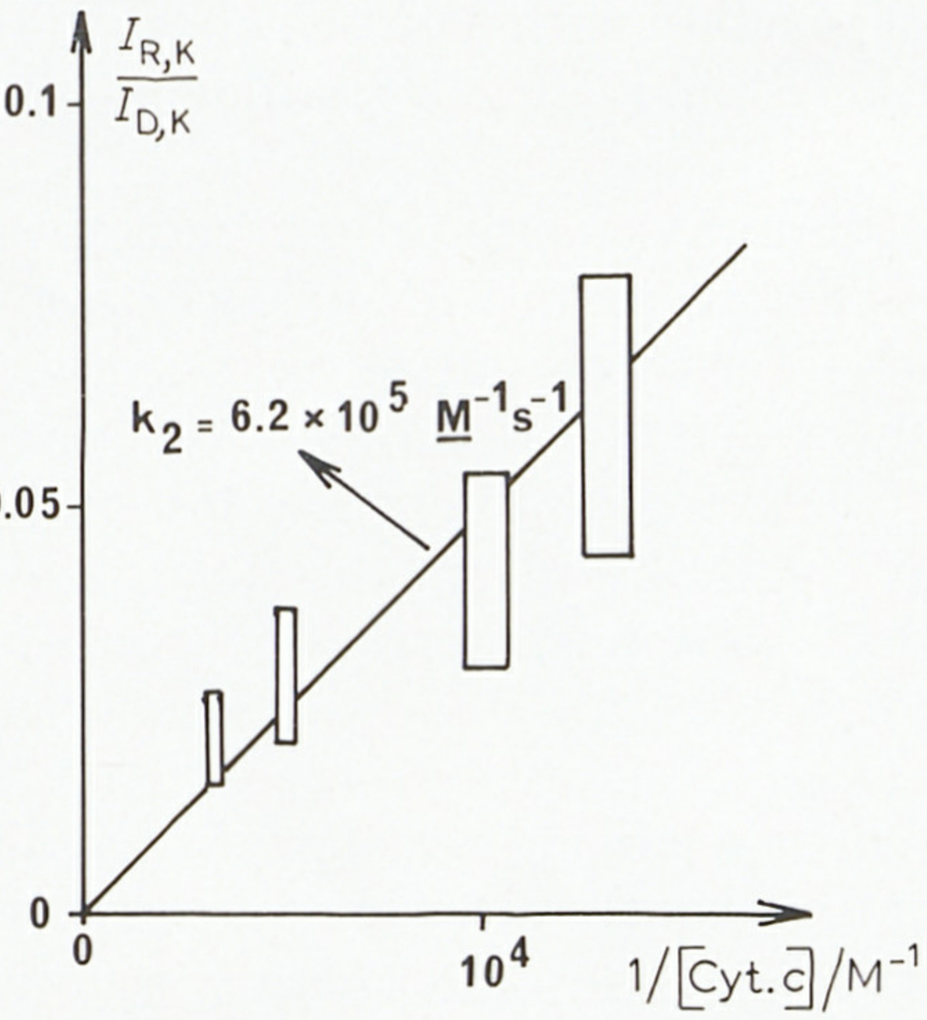

Fig. 8. Determination of the rate of the reaction between horse heart ferricytochrome *c* and $MV^{\bullet+}$. 10 m*M* MV^{2+} and 77—310 μM ferricytochrome *c*, solution buffered at pH 6.8 (0.2 *M* phosphate), electrode No. 1, 23.8 Hz, 20°C.

310 μM. Figure 8 shows that $I_{R,K}/I_{D,K}$ is proportional to 1/[C] and so $(1/[C] \times (I_{D,K}/I_{R,K})$ is constant; the value of this ratio in eqn. (c) gives the rate constant of the reaction of ferricytochrome *c* with oxygen:

$k_2 = (6.2 \pm 2)10^5\ M^{-1}\,s^{-1}$.

(5) CONCLUSION

In this investigation, the study of the reactivity of $V^{\bullet+}$ with oxygen and horse-heart ferricytochrome *c* was conducted with ring-disc electrodes in order to check the possibilities of this technique both for analytical and kinetic applications.

The first aim was partially reached since ferricytochrome *c* may be very easily titrated but oxygen gives some artefacts on the I_R versus I_D curves. The limits of sensitivity and accuracy of the method were not checked for our equipment was deficient for regulating very low oxygen concentrations. Nevertheless, we think that such titration of ferricytochrome *c* may be useful when spectroscopical determinations are impossible: it may be performed within some minutes and give a concentration with a 7% accuracy in the 20—300 μM range; when only small amounts of compound are available, titrations can be made with 2—5 ml solution in a miniaturized equipment [16].

The second aim of this work concerning kinetic measurements was only partially reached. It has not been possible to obtain an estimate of the reaction rate between oxygen and $V^{\bullet+}$ but we have determined the rate of the reaction between horse-heart ferricytochrome *c* and electrogenerated methylviologen

cation radical assumed to be second order: $(6.2 \pm 2) \times 10^5\ M^{-1}\ s^{-1}$. This result is in good agreement with the results of Kuwana [1,2] and Wilson [3] i.e. $5—10 \times 10^5\ M^{-1}\ s^{-1}$, but disagrees with the most recent value reported by Kuwana [6], i.e. more than $2 \times 10^8\ M^{-1}\ s^{-1}$. It seems quite reliable since this ring-disc determination is very simple and does not need, unlike the spectroelectrochemical method, digital simulation of the absorbance-time profiles, deconvolution of the overlapping spectra of ferrocytochrome *c* and $MV^{\bullet+}$, and signal averaging because of the small optical absorbance change at short times. The use of stationary conditions is obviously the main advantage of the use of a ring-disc electrode compared to the optical transparent electrode; both electrodes allow the generation of reactive species (either reductants or oxydants) and the study of how they react with compounds present in the bulk of the solution; they may be applied as a kinetic tool for the study of electron transfer between chemicals.

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